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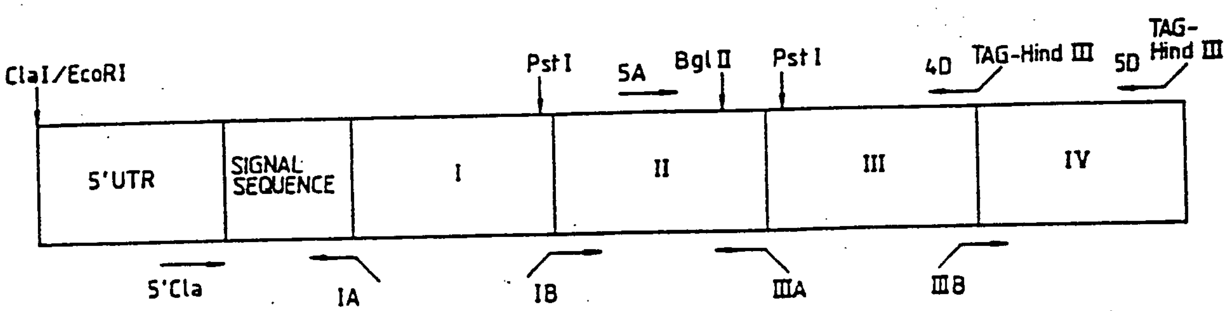

(57) Abstract

A polypeptide which is capable of binding human TNFα and which consists essentially of: a) the first three cysteine-rich subdomains, but not the fourth cysteine-rich subdomain, of the extracellular binding domain of the 55kD or 75kD receptor for human TNFα; or b) an amino acid sequence having a homology of 90 % or more with the said sequence (a).

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Modified human TNFalpha(Tumor Nec:.sis Factor alpha) Receptor.

The present invention relates to recombinant proteins and their use.

Tumour necrosis factor-α (TNFα) is a potent cytokine which elicits a broad spectrum of biological responses. TNFα causes the cytolysis or cytostasis of many tumour cell lines *in vitro*, induces the haemorrhagic necrosis of transplanted tumours in mice, enhances the phagocytosis and cytotoxicity of polymorphonuclear neutrophils, and modulates the expression of many proteins, including lipoprotein lipase, class I antigens of the major histocompatibility complex, and cytokines such as interleukin 1 and interleukin 6. TNFα appears to be necessary for a normal immune response, but large quantities produce dramatic pathogenic effects. TNFα has been termed "cachectin" since it is the predominant factor responsible for the wasting syndrome (cachexia) associated with neoplastic disease and parasitemia. TNF is also a major contributor to toxicity in gram-negative sepsis, since antibodies against TNF can protect infected animals.

The many activities of TNFα are mediated by binding to a cell surface receptor. Radioligand binding studies have confirmed the presence of TNF receptors on a wide variety of cell types. Although these receptors are expressed in limited numbers (1,000 - 10,000 receptors/cell), they bind TNFα with high affinity (Ka = $10^9 M^{-1}$ at 4°C). Lymphotoxin (LT, also termed TNFβ) has similar, if not identical, biolcgical activities to TNFα, presumably because both are recognized by the same receptor.

Recently, several laboratories have detected heterogeneity in TNF receptor preparations. Two distinct cell surface receptors which bind TNFα and TNFβ have recently been characterised at the molecular level. cDNA for one form of the receptor with a Mr of 55kD was isolated utilising probes designed from the peptide sequence of a

soluble form of the receptor (1,2). A second receptor of Mr 75kD was cloned by a COS cell expression approach (3). Both receptors are members of a larger family of cytokine receptors which include the nerve growth factor receptor, the B cell antigen CD40, the rat T cell antigen MRC OX40. In addition these receptors are homologous to the predicted product of a transcriptionally active open reading frame from shope fibroma virus which appears to give rise to a secreted protein.

The most conserved feature amongst this group of cell surface receptors is the cysteine rich extracellular ligand binding domain, which can be divided into four repeating motifs of about forty amino acids. We have now generated four soluble receptor derivatives of the 55kD TNFα receptor (TNFR). Each derivative is composed of the extracellular binding domain but without one of the cysteine rich subdomains. We have found that the derivative which lacks the membrane-proximal fourth subdomain retains the ability to bind TNFα with high affinity. This finding has general applicability.

Accordingly, the present invention provides a polypeptide which is capable of binding human TNFα and which consists essentially of:

(a) the first three cysteine-rich subdomains, but not the fourth cysteine-rich subdomain, of the extracellular binding domain of the 55kD or 75kD receptor for human TNFα; or

(b) an amino acid sequence having a homology of 90% or more with the said sequence (a).

The invention also provides:

- a DNA sequence which encodes such a polypeptide;
- a vector which incorporates a DNA sequence of the invention and which is capable, when provided in a transformed host, of expressing the polypeptide of the invention encoded by the DNA sequence; and

a host transformed with such a vector.

In the accompanying drawings:

Figure 1 shows the nucleotide sequence of the human TNFα cDNA and encoded amino acid sequence. The predicted signal sequence residues are numbered -40 to -1. The transmembrane domain is boxed and potential N-linked glycosylation sites are overlined. The sequence homologous with the designed oligonucleotide probe is found at nucleotide positions 477-533.

Figure 2 is a Northern blot (lanes 1-3) of 10μg of oligo-dT selected RNA from human 293 cells (fibroblast cell line) (lane 1), placenta (lane 2) and spleen (lane 3) hybridised with the TNF receptor cDNA (Sma1-EcoRI fragment). The Southern blot (lanes 4-6) was hybridized with the same probe. Human genomic DNA (5 μg per lane) was digested with Pst1 (lane 4), Hind III (lane 5) and EcoRI (lane 6).

Figure 3 shows the binding characteristics of recombinant human TNF receptor expressed in COS-7 cells. The direct binding of recombinant ^{125}I-TNFα to COS-7 cells transfected with prTNFR is presented in panel A. The inset contains Scatchard analysis derived from this data. As shown in panel B, monolayers of Cos-7 cells transfected with TNFR cDNA were incubated with 1nM ^{125}I-TNF in the presence of various concentrations of unlabelled TNFα or TNFβ.

Figure 4 shows the effects of soluble TNFR on TNFα binding and biological activity. Panel A shows the effects of supernatants from Cos-7 cells transfected with a cDNA encoding a soluble form of the TNF receptor (pTNFRecd, closed circles) or mock transfected (open circles) on ^{125}I-TNF binding to U937 cells. Panel B shows the effects of these supernatants on TNF mediated killing of WEHI 164 (clone 13) line. Assays were performed as described in Materials and Methods.

Figure 5 is a diagram of the DNA sequence of pTNFRecd and is also a strategy map for polymerase chain reaction (PCR)-based domain deletion, in which 5'UTR is the 5'-untranslated region and I to IV are the four cysteine-rich subdomains. The oligonucleotides employed in PCR in the Example and relevant restriction sites are also shown.

Figure 6 shows lined up the amino acid sequences of the four cysteine-rich subdomains of the 55kD (TNFR-55) and 75kD (TNFR-75) receptors and of rat nerve growth factor receptor (NGFR), human CD40 and rat OX40. Homology is shown by means of boxes.

Figures 7 to 11 show the nucleotide sequence and the predicted amino acid sequence of the encoded polypeptide of pTNFRecd, pΔI, pΔII, pΔIII and pΔIV.

Figure 12 shows the results of the assays described in the Example 1.

Figure 13 shows diagrammatically the DNA encoding the 75kD receptor in which I to IV are the four cysteine-rich subdomains. Oligonucleotides employed in PCR-domain deletion are also shown.

A polypeptide according to the invention is capable of binding human TNFα. Typically the polypeptide has a binding affinity for human TNFα of $10^7 M^{-1}$ or greater, for example $10^8 M^{-1}$ or greater. The affinity may be from 10^7 to $10^{10}\ M^{-1}$, for example from 10^8 to $10^9 M^{-1}$.

A preferred polypeptide consists essentially of the first three cysteine-rich subdomains of the extracellular binding domain of the 55kD receptor for human TNFα. The sequence (a_1) of these three subdomains is: V C P Q G K Y I H P Q N N S I C C T K C H K G T Y L Y N D C P G P G Q D T D C R E C E S G S F T A S E N H L R H C L S C S K C R K E M G Q V E I S S C T V D R D T V C G C R K N Q Y R H Y W S E N L F Q C F N C S L C L N G T V H L S C Q E K Q N T V C.

A useful polypeptide has the amino acid sequence (c):

M G L S T V P D L L L P L V L L E L L V
G I Y P S G V I G L V P H L G D R E K R
D S V C P Q G K Y I H P Q N N S I C C T
K C H K G T Y L Y N D C P G P G Q D T D
C R E C E S G S F T A S E N H L R H C L
S C S K C R K E M G Q V E I S S C T V D
R D T V C G C R K N Q Y R H Y W S E N L
F Q C F N C S L C L N G T V H L S C Q E
K Q N T V C T.

In an alternative embodiment, the polypeptide may consist essentially of the first three cysteine-rich subdomains of the extracellular binding domain of the 75kD receptor.

Apart from the amino acid sequence (a), the polypeptides may alternatively consist essentially of an amino acid sequence (b) having a homology of 90% or more with sequence (a). The degree of homology may be 95% or more or 98% or more. Amino acid sequence (a) may therefore be modified by one or more amino acid substitutions, insertions and/or deletions and/or by an extension at either or each end. There should be no modification of the cysteine-residues, however. A polypeptide comprising sequence (b) must of course still be capable of binding human TNFα.

For example, one or more amino acid residues of the sequence (a), other than a cysteine residue, may be substituted or deleted or one or more additional amino acid residues may be inserted; provided the physicochemical character of the original sequence is preserved, i.e. in terms of charge density, hydrophobicity/hydrophilicity, size and configuration. Conservative substitutions may be made. Candidate substitutions are, based on the one-letter code (Eur. J. Biochem. <u>138</u>, 9-37, 1984):

A for G and *vice versa*,

V by A, L or G;
K by R;
S by T and _vice versa_;
E for D and _vice versa_; and
Q by N and _vice versa_.

Up to 15 residues may be deleted from the N-terminal and/or C-terminal of the polypeptide, for example up to 11 residues or up to 5 residues.

The polypeptides of the invention consist essentially of sequence (a) or (b). They do not contain a fourth cysteine-rich subdomain. However, the polypeptides may be longer polypeptides of which sequence (a) or (b) is a part. A short sequence of up to 50 amino acid residues may be provided at either or each terminal of sequence (a) or (b). The sequence may have up to 30, for example up to 20 or up to 10, amino acid residues.

Alternatively, a much longer extension may be present at either or each terminal of sequence (a) or (b) of up to, for example, 100 or 200 amino acid residues. Longer amino acid sequences may be fused to either or each end. A chimaeric protein may be provided in which the or each extension is a heterologous amino acid sequence, i.e. a sequence not naturally linked to the amino acid sequence above. Such a chimaeric protein may therefore combine the ability to bind specifically to human TNFα with another functionality.

The polypeptides of the invention lack the fourth cysteine-rich subdomain of the 55kD or 75kD receptor as the case may be. In particular, they lack the cysteine residues of the fourth subdomain. They therefore do not comprise, immediately after the third cysteine-rich subdomain, any of the amino acid sequence up to the last cysteine residue of the fourth cysteine-rich subdomain of the relevant receptor except possibly the first amino acid residue of that sequence. The polypeptides may extend

beyond that first amino acid residue as indicated above, though, by way of other amino acid sequences.

The polypeptides are typically recombinant polypeptides, although they may be made by synthetic methods such as solid-phase or solution-phase polypeptide synthesis in which case an automated peptide synthesiser may be employed. They may therefore commence with a N-terminal residue M. They are prepared by recombinant DNA technology. The preparation of the polypeptides therefore depends upon the provision of a DNA sequence encoding the polypeptide. A suitable sequence encoding the first three cysteine-rich subdomains of the extracellular binding domain of the 55kD receptor comprises: GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC.

A DNA sequence may further comprise a DNA sequence encoding a signal sequence fused to the 5' end of the coding sequence. Any signal sequence may be appropriate. The signal sequence should be capable of directing secretion of the polypeptide of the invention from the cell in which the polypeptide is expressed. The signal sequence may be the natural signal sequence for the 55kD TNFα receptor. An appropriate DNA sequence encoding the first three cysteine-rich subdomains of the extracellular binding domain of the 55kD receptor and such a signal sequence is therefore: ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT

TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC.

A DNA sequence encoding a polypeptide of the invention may be synthesised. Alternatively, it may be constructed by isolating a DNA sequence encoding the 55kD or 75kD receptor from a gene library and deleting DNA downstream of the coding sequence for the first three cysteine-rich subdomains of the extracellular binding domain of the receptor. This gives DNA encoding the first three subdomains of either receptor. As an intermediate step, DNA encoding the entire or nearly the entire extracellular binding domain may be isolated and digested to remove DNA downstream of the coding sequence for the first three subdomains.

A modified nucleotide sequence, for example encoding an amino acid sequence (b), may be obtained by use of any appropriate technique, including restriction with an endonuclease, insertion of linkers, use of an exonuclease and/or a polymerase and site-directed mutagenesis. Whether a modified DNA sequence encodes a polypeptide of the invention can be readily ascertained. The polypeptide encoded by the sequence can be expressed in a suitable host and tested for its ability to bind specifically human TNFα.

For expression of a polypeptide of the invention, an expression vector is constructed. An expression vector is prepared which comprises a DNA sequence encoding a polypeptide of the invention and which is capable of expressing the polypeptide when provided in a suitable host. Appropriate transcriptional and translational

control elements are provided, including a promoter for the DNA sequence, a transcriptional termination site, and translational start and stop codons. The DNA sequence is provided in the correct frame such as to enable expression of the polypeptide to occur in a host compatible with the vector.

The expression vector is then provided in an appropriate host. Cells harbouring the vector are grown so as to enable expression to occur. The vector may be a plasmid or a viral vector. Any appropriate host-vector system may be employed.

The transformed host may be a prokaryotic or eukaryotic host. A bacterial or yeast host may be employed, for example _E. coli_ or _S. cerevisiae_. Insect cells can alternatively be used, in which case a baculovirus expression system may be appropriate. As a further alternative, cells of a mammalian cell line, such as Chinese Hamster Ovary (CHO) Cells may be transformed. A polypeptide glycosylated at one, two or three of the sites shown in Figure 1 can be obtained by suitable choice of the host cell culture.

The polypeptide of the invention can be isolated and purified. The N-terminal of the polypeptide may be heterogeneous due to processing of the translation product within a cell or as the product is being secreted from a cell. A mixture of polypeptides according to the invention, having different N-terminii, may therefore be obtained. The polypeptide is soluble.

The polypeptides of the invention have activity binding human TNFα. This activity is indictive of the possible use of the polypeptides in the regulation of TNFα-mediated responses by binding and sequestering human TNFα, for example possible use in treatment of pulmonary diseases, septic shock, HIV infection, malaria, viral meningitis, graft versus host reactions and autoimmune diseases such as

rheumatoid arthritis.

For this purpose, a polypeptide of the present invention may be formulated in a pharmaceutical composition. The pharmaceutical composition also comprises a pharmaceutically acceptable carrier or diluent.

The polypeptide of the invention may be administered to a patient by any convenient route. The choice of whether an oral route or a parenteral route, such as subcutaneous, intravenous or intramuscular administration, is adopted; of the dose; and of the frequency of administration depends upon a variety of factors. These factors include the purpose of the administration, the age and weight of the patient being treated and the condition of the patient. Typically, however, the polypeptide is administered in an amount of from 1 to 1000 μg per dose, more preferably from 10 to 100 μg per dose, for each route of administration.

The following Examples illustrate the invention. A Reference Example is provided.

<u>REFERENCE EXAMPLE</u>

1. <u>Materials and Methods</u>

<u>Reagents</u>

Recombinant human TNFα and TNFβ were supplied as highly purified proteins derived from *E. coli*. The specific activities of these preparations were approximately 10^7 units/mg, as measured in the murine L929 cell cytotoxicity assay (4). The synthetic oligonucleotides were prepared by Oswel DNA Service (University of Edinburgh).

<u>Isolation of TNFα 55kD receptor cDNA clones</u>

The sequence of a peptide fragment (E M G Q V E I S S T V D R D T V C G) of the TNF binding protein was used to design a synthetic oligonucleotide probe (5' AAG GAG ATG GGC CAG GTT GAG ATC TCT TCT ACT GTT GAC AAT GAC ACT GTG TGT GGC-3'). The 57-mer DNA probe was labelled with ^{32}P and T4

polynucleotide kinase (New England Biolab, Beverly, MA) and used to screen a placenta cDNA library in gt10 (5,6). Approximately 800,000 phage were transferred to nitrocellulose filters and screened at reduced stringency (7). Filters were incubated for 2 hours at 42°C in 0.05M sodium phosphate, pH 6.5, 20% formamide, 0.75 M sodium chloride, 0.075 M sodium citrate, 1% polyvinyl pyrrolidone (Sigma, St Louis, MO), 1% Ficoll, 1% bovine serum albumin (Sigma), and 50 ng/ml sonicated salmon sperm DNA (Sigma). The radiolabelled probe was then added to the filters (10^8 cpm/ml final concentration) which were hybridized for 16 hours. Filters were washed extensively in 0.06M sodium chloride, 0.006M sodium citrate, 1% SDS at 37°C and positive clones were identified by autoradiography. Ten hybridizing clones were plaque purified (5) and cDNA insert size was determined by polyacrylamide gel electrophoresis of EcoRI digested phage DNA. The inserts of two cDNA clones were sequenced using the dideoxy chain termination technique (8).

<u>Southern and Northern blot analysis</u>

DNA was isolated from human lymphocytes by the method of Blin and Stafford (9) and used for Southern blot analysis (10). DNA was digested with restriction endonucleases (New England Biolabs), fractionated on a 1% agarose gel, and transferred to nitrocellulose. Hybridization and washing were conducted under stringent conditions (6) using a ^{32}P-labelled preparation of a 600 bp fragment of the TNF receptor cDNA. Northern blot analysis was performed (11) on oligo-dT selected RNA isolated from human placenta, spleen (generously provided by the Cooperative Human Tissue Network, Birmingham, AL) and a fibroblast cell line (293 cells). Following electrophoresis on a formaldehyde 1.2% agarose gel, the RNA was transferred to nitrocellulose and hybridized with the TNFα receptor DNA probe under stringent conditions.

Mammalian cell expression of the human TNFα 55kD receptor and derivatives

The coding region of the majority of the human TNFα 55kD receptor was isolated as an EcoRI fragment and cloned into a mammalian cell expression vector (12), resulting in plasmid prTNFR. The EcoRI fragment encodes 374 amino acids of the TNF receptor; the 81 carboxyl terminal residues of the cytoplasmic domain are therefore missing from this plasmid construction. A derivative of the TNFα receptor was produced by engineering a termination codon just prior to the transmembrane domain. The polymerase chain reaction (PCR) technique (13) was used to generate a 300 bp restriction fragment containing a BgIII site at the 5' end and a HindIII site preceded by a TAG stop codon at the 3' end. The PCR primers were 5'GCTGCTCCAAATGCCGAAAG and 5'AGTTCAAGCTTTTACAGTGCCCTTAACATTCTAA.

The PCR product was gel purified and cloned into the TNF receptor expression plasmid (described above) digested with BgIII and HindIII. DNA sequencing confirmed that the resulting plasmid (pTNFRecd) contained the designed DNA sequence. E. coli harbouring pTNFRecd were deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, GB on 11 September 1990 under accession number NCIMB 40315.

The TNFα receptor expression plasmids were transfected into monkey COS-7 cells using Lipofectin (Gibco BRL, Bethesda, MD) according to the manufacturer's instructions. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Analysis of recombinant TNFα 55kD receptor derivatives

TNFα was radioiodinated with the Iodogen method (Pierce) according to the manufacturer's directions. The specific activity of the ^{125}I-TNFα was 10-30 μCu/μg. COS cells

transfected with the TNFα receptor cDNA (prTNFR, 1300 bp EcoRI fragment) were incubated for 24 hours and then seeded into six well tissue culture plates (Nunc) at 4.5 x 10^8 cells per well. The cells were incubated for a further 48 hours and then receptor expression was quantitated by radioligand binding for 2 hours at 4°C. Non-specific binding of ^{125}I-TNFα was determined in the presence of a 1,000 fold molar excess of unlabelled TNFα. Binding data was analysed by the method of Scatchard (14).

The TNFα receptor derivative was analysed for inhibition of ^{125}I-TNFα binding to the natural receptor on human U937 cells. Culture supernatant was harvested 72 hours after COS cells were transfected with pTNFRecd. U937 cells (2 x 10^8 cells in 200 µl) were incubated with 1nM ^{125}I-TNFα and dilutions of COS cell media for 2 hours at 4°C. Cells were then centrifuged through 20% sucrose to remove unbound TNFα. Non-specific binding was determined in the presence of 1µM unlabelled TNFα.

The TNFα receptor derivative was also analyzed for inhibition of TNFα cytotoxic effects <u>in vitro</u>. The cytotoxicity assay was performed as described on the TNF sensitive cell line WEHI 164 clone 13 (15). Serial dilutions of supernatants from COS cells transfected with pTNFRecd or mock transfected controls were incubated with a constant amount of TNFα (1 ng/ml) for 1 hour at 27°C before addition to the assay.

2. RESULTS

Isolation and characterization of the TNFα 55kD receptor cDNA

A partial amino acid sequence of the TNF binding protein was used to design a synthetic oligonucleotide probe. The radiolabelled probe was used to screen a human placenta cDNA library in lambdagt10 and ten hybridizing phage were isolated. The nucleotide and deduced amino acid sequences

of the longest cDNA clone are depicted in Figure 1. The third potential ATG initiation codon occurs at position 156 of the nucleotide sequence; the first two ATG codons are closely followed by termination codons, and the third ATG is preceded by the best translation initiation consensus nucleotides (16). The cDNA encodes an open reading frame of 1365 bases which codes for a polypeptide of 455 residues. Both of the peptide sequences determined by amino acid sequencing were identified in the encoded cDNA (17 of 19 and 18 of 19 matching residues). The amino terminal end identified for the TNF binding protein corresponds to the cDNA encoded sequence beginning at residue 41. The first 35 amino acids are generally quite hydrophobic and probably represent a signal sequence. Residues 35-40 are highly charged (DREKR) and such a sequence is not typically found in secretory signal sequences (17); perhaps the natural receptor is processed by proteolysis after residue 40 which contains a dibasio cleavage site (KR). Hydropathy analysis of the protein sequence predicts a single transmembrane domain of 23 amino acids. This hydrophobic sequence divides the protein into an extracellular domain of 171 residues and a cytoplasmic domain of 221 residues. The amino acid composition determined for the TNF binding protein corresponds well with the predicted composition of the extracellular domain encoded by the cDNA (results not shown). The discrepancy between the predicted receptor size (40,000 daltons) and the size determined by SDS-polyacrylamide gel electrophoresis (65,000 daltons, 18-20) is probably due to glycosylation; there are four potential N-linked glycosylation sites in the sequence, three of which are in the extracellular domain. The sequence contains a large number (17) of cysteine residues, 24 of which are in the extracellular domain. The arrangement of these cysteine residues is similar to that of several other cell surface

proteins, suggesting that the TNF receptor is structurally related to a family of receptors.

A Northern blot analysis is presented in Figure 2. The ^{32}P-labelled cDNA hybridized to a single predominant band of oligo-dT selected RNA from human placenta or spleen. A minor larger transcript was also observed in RNA from a fibroblast cell line. The size of the hybridizing species is 2400 bases, in good agreement with the size of isolated cDNA. Also shown in Figure 2 is a Southern blot of human genomic DNA hybridized with a 600 bp probe from the cDNA. In each of the three different restriction digests, only a single hybridized signal was observed. Thus the TNF receptor that we have isolated appears to be encoded by a single gene.

Expression of recombinant TNF receptor sequences in mammalian cells

To confirm that the cDNA shown in Figure 1 indeed encodes the TNF receptor, the cDNA was engineered for expression in mammalian cells. The cDNA contains an EcoRI site at position 1270 of Figure 1. The receptor coding sequence was isolated as a 1300 bp EcoRI-fragment (containing all but the last 81 codons of the cytoplasmic domain) and inserted into a mammalian cell expression vector containing a cytomegalovirus promoter and SV40 transcription termination sequences (12). The resulting plasmid was transfected into COS cells which were analyzed for TNF receptor expression after three days. As shown in Figure 3, the transfected cells specifically bound radioiodinated TNFα in a saturable and dose dependent fashion. The population of COS cells expressed approximately 1×10^8 receptors per cell. The measured binding affinity of recombinant receptors was $2.5 \times 10^9 M^{-1}$ at 4°C which is in close agreement with natural receptor on human cells (19,20). The binding of ^{125}I-TNFα(1 nM) to

these cells could be inhibited by the addition of unlabelled TNFα or lymphotoxin (Figure 3b). COS cells transfected with just the expression vector did not significantly bind ^{125}I-TNFα (less than 2% of the binding seen with the cDNA transfection).

The extracellular domain of the TNF receptor is naturally shed from cells. To produce a similar recombinant derivative, a stop codon preceding the transmembrane domain was engineered into the cDNA by PCR mutagenesis. The modified DNA was inserted into the expression plasmid and subsequently transfected into COS cells. After three days, the COS cell media was tested for inhibition of TNFα binding to human U937 cells. As shown in Figure 4a, the transfected cell media inhibited up to 70% of the binding of TNFα. The recombinant TNF receptor derivative was next tested for inhibition of TNFα biological activity. A sensitive bioassay for TNFα is a measurement of cytolysis of mouse WEHI 164 (clone 13) cells. The transfected cell media inhibited 60% of TNFα cytotoxicity on this cell line (Figure 4b). Media from mock transfected COS cells did not inhibit TNFα induced cytotoxicity or binding. These experiments demonstrate that the recombinant extracellular domain of the TNF receptor is capable of binding TNF and inhibiting its biological activity.

<u>EXAMPLE 1: Expression of polypeptide consisting essentially of the first three cysteine-rich subdomains of the extracellular binding domain of the 55kD receptor</u>

1. <u>MATERIALS AND METHODS</u>

<u>Reagents</u>

<u>E. coli</u> derived recombinant human TNFα had a specific activity of 2 x 10^7 U/mg in an L929 cytotoxicity assay. Oligonucleotides were purchased from Oswel DNA service (University of Edinburgh).

Generation of the recombinant soluble TNFR derivatives

Deletion of each of the subdomains in the recombinant soluble TNFR was achieved by means of PCR fragment joining and PCR mutagenesis. The sequence of the oligonucleotides used in these experiments is given in Table 1 and their locations relative to the four cysteine rich subdomains is shown in Figure 5. The four subdomains are lined up with respect to one another in Figure 6.

The plasmid pTNFRecd (Reference Example) is shown in Figure 7. pTNFRecd was further modified to remove 5' untranslated sequences by cloning of the Cla I/Bgl II digested product of a PCR using oligos 5' Cla and IIIA into ClaI/Bgl II digested pTNFRecd, to generate 5'-ΔCla. Digestion of 5'-ΔCla with Pst-1 and religation resulted in the generation of pΔII, which lacks the second cysteine rich subdomain (Figure 9). The fourth cysteine rich subdomain was removed by cloning of the BglII/Hind III digested product of a PCR using oligonucleotides 5A and 4D into BglII/Hind III 5'-ΔCla; this introduced a termination codon after amino acid 167 (counting from the initial methionine) to yield pΔIV (Figure 11). The constructs p I (Figure 8) and pΔIII (Figure 10) which lack the first and third cysteine rich subdomains respectively were generated by joining PCR fragments by means of overlaps introduced into the primers used for the PCR. The gel purified products of PCR's using 5' Cla and IA and IB and 5D were mixed and subjected to further amplification using 5'Cla and 5D as primers. The resulting fragment was digested with ClaI and BglII and cloned into ClaI/BglII digested pTNFRecd, to yield pΔI.

Similarly the gel purified products of PCR's using 5' Cla and IIIA and IIIB and 5D were mixed and subjected to further amplification using 5'Cla and 5D as primers. This product was digested with BglII and HindIII and cloned into Bgl II/Hind III cut 5'-Δ Cla to yield pΔIII. In all cases

the cloned derivatives were analysed by restriction enzyme analysis and DNA sequencing using sequenase (United States Biochemical Corporation).

Table 1: Structure of the mutagenic oligonucleotides

Oligo Name	Sequence
5'Cla	5'-GTTCTATCGATAAGAGGCCATAGCTGTCTGGC-3'
IA	5'-GCTCTCACACTCTCTTCTCCCTGTCCCCTAG-3'
IB	5'-AGGGAGAAGAGAGAGTGTGAGAGCGGCTCCTTC-3'
IIIA	5'-TGCATGGCAGGTACACACGGTGTCCCGGTCCAC-3'
IIIB	5'-GACACCGTGTGTACCTGCCATGCAGGTTTCTTT-3'
4D	5'-GGCCAAGCTTCAGGTGCACACGGTGTTCTG-3'
5A	5'-GCTGCTCCAAATGCCGAAAG-3'
5D	5'-AGTTCAAGCTTTACAGTGCCCTTAACATTCTAA-3'

Analysis of recombinant soluble TNFR derivatives

COS cells were maintained in Dulbecco's modified Eagles medium containing 5% foetal calf serum. The soluble TNFα receptor derivatives were transfected into monkey COS cells by means of lipofectin (GIBCO-BRL, Bethesda MD) according to the manufacturers protocol and cell free supernatants harvested 72 hours post transfection.

Inhibition of TNFα activity

The soluble TNFα receptor derivatives were analyzed for inhibition of TNFα cytotoxic activity *in vitro*. The cytotoxicity assay was performed as described on the TNFα sensitive cell line WEHI 164 clone 13. Serial dilutions of supernatants from COS cells transfected with the mutant receptors or mock transfected controls were incubated with a constant amount of TNF (1 ng/ml) for 1 hour at 37°C before addition to the assay.

2. RESULTS

In order to understand more about the contribution of

the individual cysteine rich subdomains to the binding of TNFα by the soluble form of the 55kD TNF receptor, we removed each subdomain by PCR mutagenesis (Figure 5). COS cells were transfected with each of these constructs and the supernatants were assayed for their ability to inhibit the cytotoxic activity of TNFα. Figure 12 panel A shows that conditioned medium from COS cells tranfected with pTNFRecd inhibits TNFα as previously described. Removal of the fourth cysteine rich subdomain resulted in a protein which, similar to TNFRecd, was a potent inhibitor of TNFα (Figure 12 panel B). The mutants lacking the first, second and third subdomains did not show any inhibitory activity in the TNFα cytotoxicity assay.

<u>EXAMPLE 2: Expression of polypeptide consisting essentially of the first three cysteine-rich subdomains of the extracellular binding domain of the 75kD receptor.</u>

The coding region of the human 75kD TNFα receptor was isolated from a T cell lambda ZAP library, using a probe based on published sequences (3) and cloned into the EcoRI site of a mammalian cell expression vector (12) resulting in plasmid p75TNFR. In more detail, RNA was extracted from a cell line expressing the 75kD receptor and reverse transcribed. Any cell line expressing this receptor could be used, such as those described by Smith <u>et al</u> (3). The product of the reverse transcription was subjected to 25 cycles of PCR using the following primers:

5' CGC AGA ATT CCC CGC AGC CAT GGC GCC CGT CGC C 3'
and 5' GTA AGG ATC CTA TCG CCA GTG CTC CCT TCA GCT 3'.

These primers are directed against the extracellular binding domain coding region of the 75kD receptor and were taken from Smith <u>et al</u> (3). The amplified product was gel purified and shown to encode TNFR. This was subsequently used to screen the library. Plaque purification was performed essentially as described in the Reference Example

except that the probe was labelled by random priming (21) and hybridised in 50% formamide. Filters were washed in 0.2 x SSC (Standard Saline Citrate) twice at 60°C.

A derivative of the 75kD TNFα receptor was produced by engineering a termination codon just prior to the transmembrane domain. Referring to Figure 13, the polymerase chain reaction (PCR) technique was used to generate a 274 bp restriction fragment containing a BglII site at the 5' end and an Xba I site preceded by a TAG stop codon at the 3' end. The PCR primers were 5' ACACGACTTCATCCACGGATA and 5'ACGTTCTAGACTAGTCGCCAGTGCTCCCTTCAGCTG. The PCR product was digested with Bgl II and Xba I, gel purified and cloned into the TNF receptor expression plasmid (described above) digested with BglII and Xba I. DNA sequencing confirmed that the resulting plasmid contained the designed DNA sequence.

A similar approach was utilised to generate a construct which lacked the fourth cysteine-rich subdomain of the 75kD TNFα receptor. PCR was performed using a primer upstream of the Esp I site in the 75kD TNFR and a primer which introduced a TAG termination codon and an Xba I site. The sequences of the primers was 5' CAG AAC CGC ATC TGC ACC TGC and 5'ACGTTCTAGACTTGCACACCACGTCTGATGTTTC respectively. The PCR product was digested with EspI and Xba I and the 110bp DNA fragment gel purified and cloned into Esp I Xba I digested p75TNFR.

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<u>CLAIMS</u>

1. A polypeptide which is capable of binding human TNFα and which consists essentially of:

(a) the first three cysteine-rich subdomains, but not the fourth cysteine-rich subdomain, of the extracellular binding domain of the 55kD or 75kD receptor for human TNFα; or

(b) an amino acid sequence having a homology of 90% or more with the said sequence (a).

2. A polypeptide according to claim 1, which consists essentially of the first three cysteiine-rich subdomains of the extracellular binding domain of the 55kD receptor for human TNFα.

3. A polypeptide according to claim 2, which has the amino acid sequence: M G L S T V P D L L L P L V L L E L V G I Y P S G V I G L V P H L G D R E K R D S V C P Q G K Y I H P Q N N S I C C T K C H K G T Y L Y N D C P G P G Q D T D C R E C E S G S F T A S E N H L R H C L S C S K C R K E M G Q V E I S S C T V D R D T V C G C R K N Q Y R H Y W S E N L F Q C F N C S L C L N G T V H L S C Q E K Q N T V C T.

4. A DNA sequence which encodes a polypeptide as defined in any one of the preceding claims.

5. A DNA sequence according to claim 4, which comprises:

GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC

CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC.

6. A DNA sequence according to claim 4 or 5, which further comprises a 5' sequence which encodes a signal amino acid sequence.

7. A DNA sequence according to claim 4, which is:
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TT TCT TGC ACA GTG GAC CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC.

8. A vector which incorporates a DNA sequence as claimed in any one of claims 4 to 7 and which is capable, when provided in a suitable host, of expressing the said polypeptide.

9. A vector according to claim 8, which is a plasmid.

10. A host transformed with a vector as claimed in claim 8 or 9.

11. A host according to claim 10, which is a mammalian cell line.

12. A process for the preparation of a polypeptide as defined in claim 1, which process comprises culturing a transformed host as claimed in claim 10 or 11 under such conditions that the said polypeptide is expressed.

13. A pharmaceutical composition comprising a pharmaceutically acceptable carrier or diluent and, as an

active principle, a polypeptide as claimed in claim 1.

14. A polypeptide as defined in claim 1 for use in the treatment of rheumatoid arthritis.

Fig. 1.

```
  1 ACCA GTGATCTCTA TGCCCGAGTC TCAACCCTCA ACTGTCACCC CAAGGCACTT GGGACGTCCT GGACAGACCG
 75 AGTCCCGGGA AGCCCCAGCA CTGCCGCTGC CACACTGCCC TGAGCCCAAA TGGGGGAGTG AGAGGCCATA GCTGTCTGGC

 40 M   G   L   S   T   V   P   D   L   L   L   P   L   V   L   L   E   L   L   V   G   I   Y   P
156 ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC

 16 S   G   V   I   G   L   V   P   H   L   G   D   R   E   K   R   D   S   V   C   P   Q   G   K
228 TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA

  9 Y   I   H   P   Q   N   N   S   I   C   C   T   K   C   H   K   G   T   Y   L   Y   N   D   C
300 TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT

 33 P   G   P   G   Q   D   T   D   C   R   E   C   E   S   G   S   F   T   A   S   E   N   H   L
372 CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC

 57 R   H   C   L   S   C   S   K   C   R   K   E   M   G   Q   V   E   I   S   S   C   T   V   D
444 AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC

 81 R   D   T   V   C   G   C   R   K   N   Q   Y   R   H   Y   W   S   E   N   L   F   Q   C   F
516 CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC

105 N   C   S   L   C   L   N   G   T   V   H   L   S   C   Q   E   K   Q   N   T   V   C   T   C
558 AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC

129 H   A   G   F   F   L   R   E   N   E   C   V   S   C   S   N   C   K   K   S   L   E   C   T
660 CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG

153 K   L   C   L   P   Q   I   E   N   V   K   G   T   E   D   S   G   T   T   V   L   L   P   L
732 AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC ACA GTG CTG TTG CCC CTG

177 V   I   F   F   G   L   C   L   L   S   L   L   F   I   G   L   M   Y   R   Y   Q   R   W   K
804 GTC ATT TTC TTT GGT CTT TGC CTT TTA TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG

201 S   K   L   Y   S   I   V   C   G   K   S   T   P   E   K   E   G   E   L   E   G   T   T   T
876 TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT
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Fig.1(cont.)

```
 225 K   P   L   A   P   N   P   S   F   S   P   T   P   G   F   T   P   T   L   G   F   S   P   V
 948 AAG CCC CTG GCC CCA AAC CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC AGT CCC GTG

 249 P   S   S   T   F   T   S   S   S   T   Y   T   P   G   D   C   P   N   F   A   A   P   R   R
1020 CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA

 273 E   V   A   P   P   Y   Q   G   A   D   P   I   L   A   T   A   L   A   S   D   P   I   P   N
1092 GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GCC TCC GAC CCC ATC CCC AAC

 297 P   L   Q   K   W   E   D   S   A   H   K   P   Q   S   L   D   T   D   D   P   A   T   L   Y
1164 CCC CTT CAG AAG TGG GAG GAC AGT GCC CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCC GCG ACG CTG TAC

 321 A   V   V   E   N   V   P   P   L   R   T   L   E   F   V   R   R   L   G   L   S   D   H   E
1236 GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAG GAA TTC GTG CGG CGC CTA GGG CTG AGC GAC CAC GAG

 345 I   D   R   L   E   L   Q   N   G   R   C   L   R   E   A   Q   Y   S   M   L   A   T   W   R
1308 ATC GAT CGG CTG GAG CTG CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG

 369 R   R   T   P   R   R   E   A   T   L   E   L   L   G   R   V   L   R   N   M   D   L   L   G
1380 CGG CGC ACG CCG CGG CGC GAG GCC ACG CTG GAG CTG CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC

 393 C   L   E   D   I   E   E   A   L   C   G   P   A   A   L   P   P   A   P   S   L   L   R
1452 TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC GCC GCG CTC CCG CCC GCG CCC AGT CTT CTC AGA TGA
1521 GGCTGCGCCC TGCGGGCAGC TCTAAGGACC GTCCTCGCAG ATCGCCTTCC AACCCCACTT TTTTCTGGAA AGGAGGGGTC
1601 CTGCAGGGGC AAGCAGGAGC TAGCAGCCGC CTACTTGGTG CTAACCCCTC GATGTACATA GCTTTTCTCA GCTGCCTGCG
1681 CGCCGCCGAC AGTCAGCGCT GTGCGCGCGG AGAGAGGTGC GCCGTGGGCT CAAGAGCCTG AGTGGGTGGT TTGCGAGGAT
1761 GAGGGACGCT ATGCCTCATG CCCGTTTTGG GTGTCCTCAC CAGCAAGGCT GCTCGGGGGC CCCTGGTTCG TCCCTGAGCC
1841 TTTTTCACAG TGCATAAGCA GTTTTTTTTG TTTTTGTTTT GTTTTGTTTT GTTTTTAAA TCAATCATGT TACACTAATA
1921 GAAACTTGGC ACTCCTGTGC CCTCTGCCTG GACAAGCAC ATAGCAAGCT GAACTGTCCT AAGGCAGGGG CGAGCACGGA
2001 ACAATGGGGC CTTCAGCTGG AGCTGTGGAC TTTTGTACAT ACACTAAAAT TCTGAAGTTA AG
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Fig. 2.

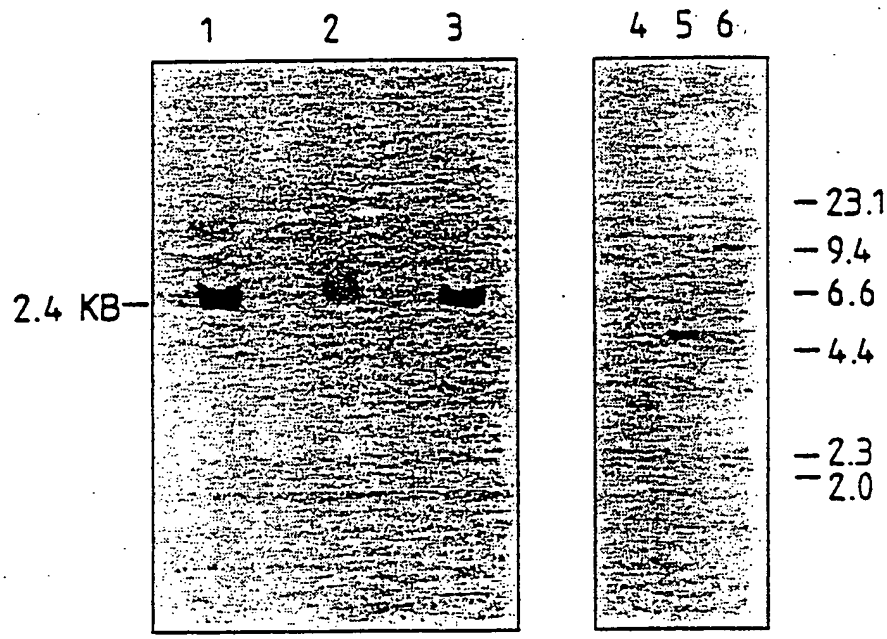


Fig. 3.

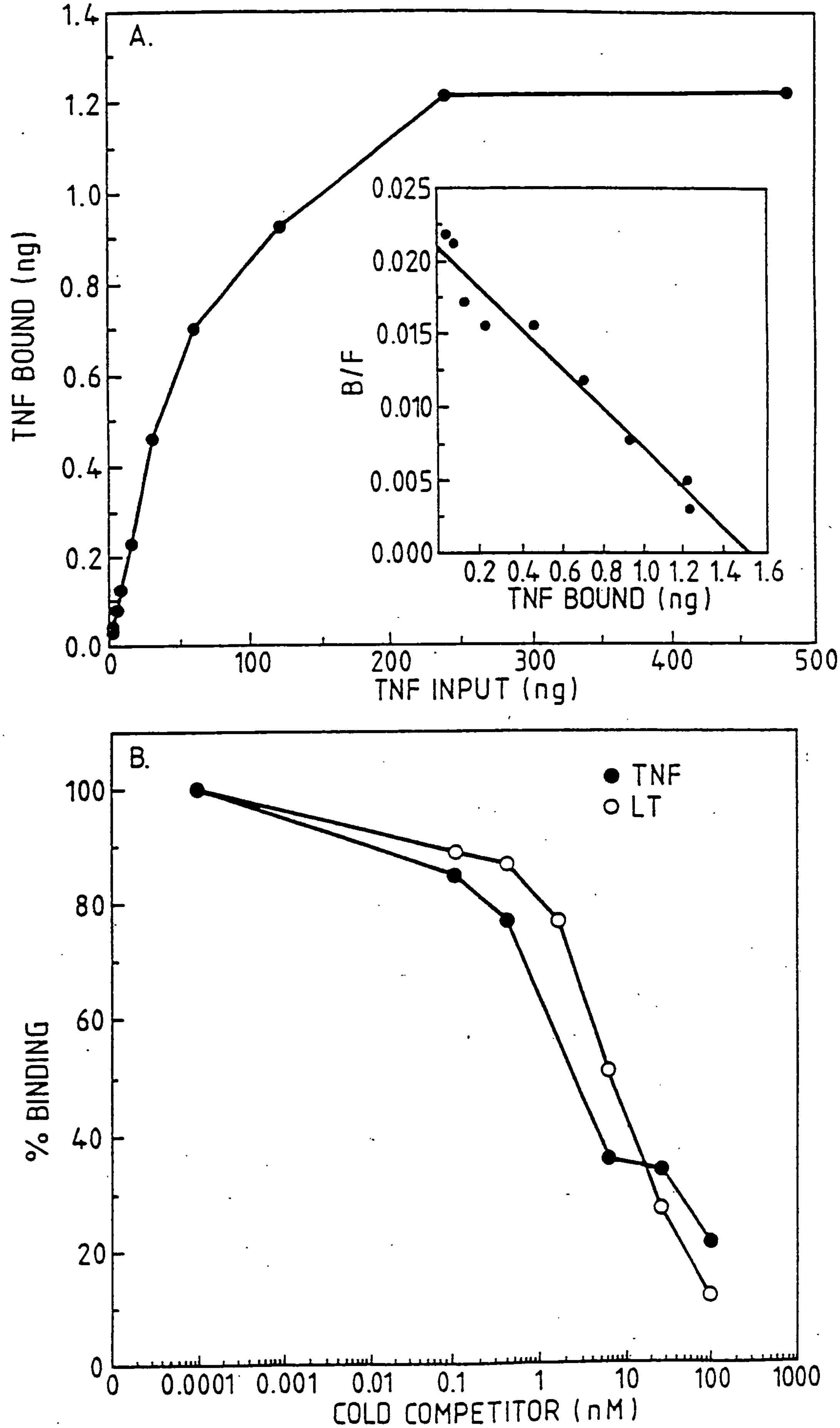

A.
TNF BOUND (ng)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
TNF INPUT (ng)
0
100
200
300
400
500
B/F
0.000
0.005
0.010
0.015
0.020
0.025
TNF BOUND (ng)
0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6
B.
TNF
LT
% BINDING
0
20
40
60
80
100
COLD COMPETITOR (nM)
0 0.0001 0.001 0.01 0.1 1 10 100 1000

Fig. 4.

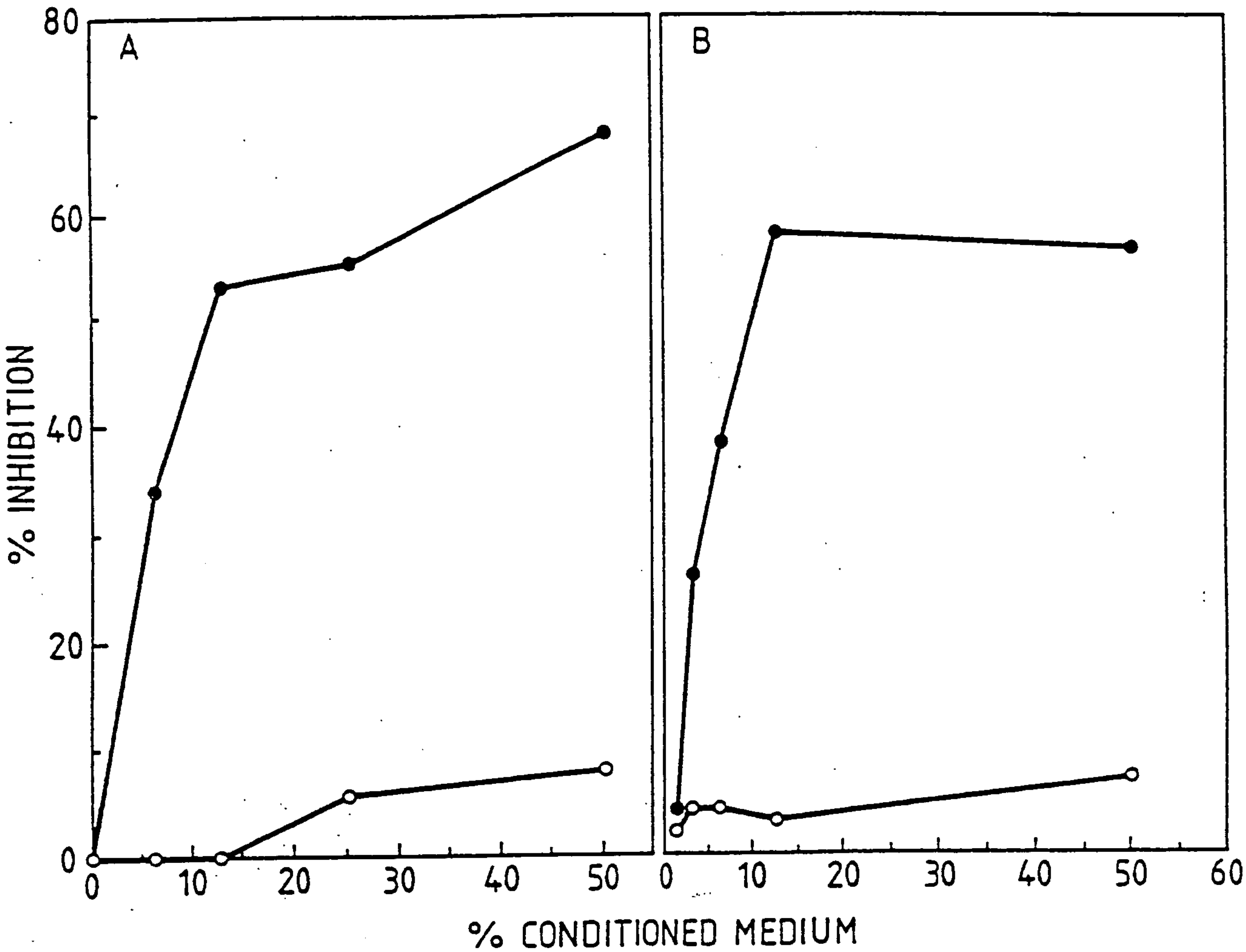

80
60
40
20
0
% INHIBITION
A
B
0
10
20
30
40
50
0
10
20
30
40
50
60
% CONDITIONED MEDIUM

Fig. 5.

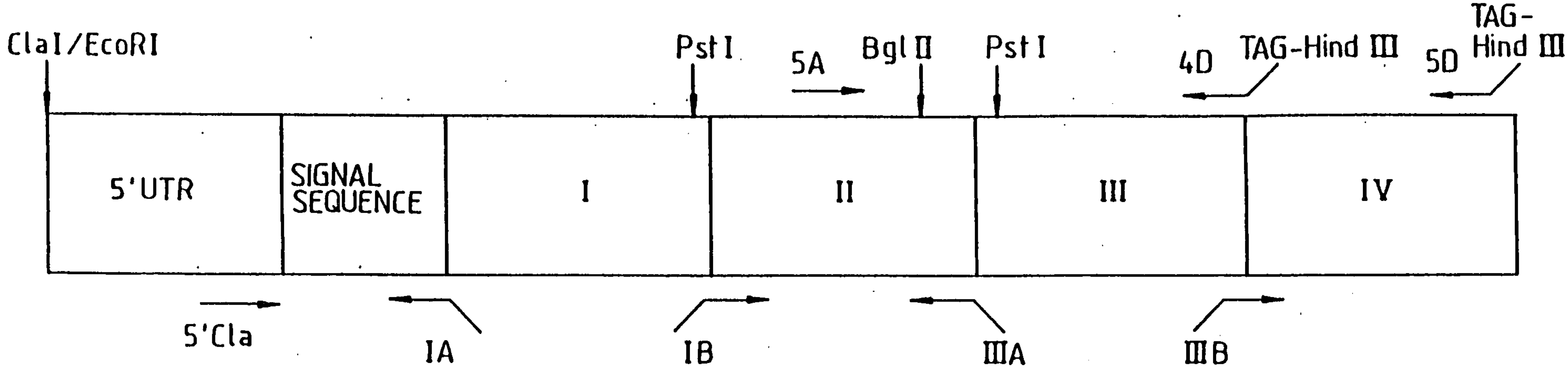

ClaI/EcoRI
PstI
5A
BglII
PstI
4D
TAG-Hind III
5D
TAG-Hind III
5'UTR
SIGNAL SEQUENCE
I
II
III
IV
5'Cla
IA
IB
IIIA
IIIB

Fig. 13.

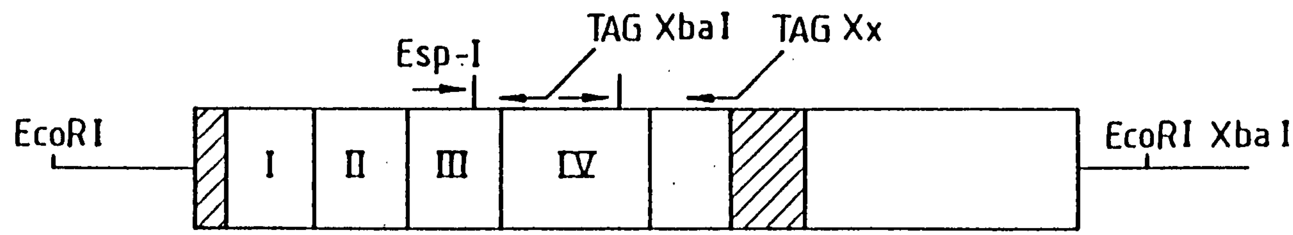

Esp-I
TAG XbaI
TAG Xx
EcoRI
I
II
III
IV
EcoRI XbaI

Fig. 6.

First Subdomain

```
TNFR-55,  V C P Q G K Y I H P Q N N S I C C T K C H K G T Y L Y N D C P G P G Q D T D C R
TNFR-75,  T C R L R E Y Y D - Q T A Q M C C S K C S P G Q H A K V F C T K T S - D T V C D
NGFR,     T C S T G L Y T H - - - S G E C C K A C N L G E G V A Q P C - G A N Q - T V C E
CD40,     A C R E K Q Y L I - - - N S Q C C S L C Q P G Q K L V S D C T E F T - E T E C L
OX40,     N C V K D T Y P S - - - G H K C C R E C Q P G H G M V S R C D H T R - D T V C H
```

Second Subdomain

```
TNFR-55,  E C - E S G S F T A S E N H L R H C L S C - S K C R K E M G Q V E I S S C T V D R D T V C
TNFR-75,  S C - E D S T Y T Q L W N W V P E C L S C G S R C - - S S D Q V E T Q A C T R E Q N R I C
NGFR,     P C L D N V T F S D V V S A T E P C K P C - T E C - - L G L Q S M S A P C V E A D D A V C
CD40,     P C G E S E F L D T W N R E - T H C H Q H - K Y C D P N L G L R V Q Q K G T S E T D T I C
OX40,     P C - E P G F Y N E A V N Y - D T C K Q C - T Q C N H R S G S E L K Q N C T P T E D T V C
```

Third Subdomain

```
TNFR-55,  G C R K N Q Y R H Y W S E N L F Q C F N C S L - - - C L N G T - V H L S C Q E K Q N T V C -
TNFR-75,  T C R P G W Y C A L S K - - Q E G C R L C A P L R K C R P G F G V A R P G T E T S D V V C K
NGFR,     R C A Y G Y Y Q D E E T - - - G H C E A C S V - - - C E V G S G L V F S C Q D K Q N T V C E
CD40,     T C E E G W H C T S E A - - - - - - C E S C V L H R S C S P G F G V K Q I A T G V S D T I C E
```

Fourth Subdomain

```
TNFR-55,  T C H A G F F L R E N - - - - E C V S C S N C K K S L E C T K L C L P Q I E N V K G T
TNFR-75,  P C A P G T F S N T T S S T D I C R P H Q I C N - - - - V V A I P G N A S M D A V C T
NGFR,     E C P E G T Y S D E A N H V D P C L P C T V C E D T E R Q L R E C T P W A - D A E C E
CD40,     P C P V G F F S N V S S A F E K C H P W T S C E T K D L V V Q Q A G T N K T D V V C G
OX40,     P C P P G H F S P G S N Q - - A C K P W T N C T L S G K Q I R H P A S N S L D T V C E
```

Fig. 7.

```
DNA   sequence    608 b.p.   TGTCTGGCATGG ... CCCCAGATTTAG   linear

9    /   1                                 39   /   11
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu leu pro leu val leu leu glu leu leu val
69   /   21                                99   /   31
GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA
gly ile tyr pro ser gly val ile gly leu val pro his leu gly asp arg glu lys arg
129  /   41                                159  /   51
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
asp ser val cys pro gln gly lys tyr ile his pro gln asn asn ser ile cys cys thr
189  /   61                                219  /   71
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC
lys cys his lys gly thr tyr leu tyr asn asp cys pro gly pro gly gln asp thr asp
249  /   81                                279  /   91
TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC
cys arg glu cys glu ser gly ser phe thr ala ser glu asn his leu arg his cys leu
309  /   101                               339  /   111
AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC
ser cys ser lys cys arg lys glu met gly gln val glu ile ser ser cys thr val asp
369  /   121                               399  /   131
CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT
arg asp thr val cys gly cys arg lys asn gln tyr arg his tyr trp ser glu asn leu
429  /   141                               459  /   151
TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG
phe gln cys phe asn cys ser leu cys leu asn gly thr val his leu ser cys gln glu
489  /   161                               519  /   171
AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC
lys gln asn thr val cys thr cys his ala gly phe phe leu arg glu asn glu cys val
549  /   181                               579  /   191
TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT TAG
ser cys ser asn cys lys lys ser leu glu cys thr lys leu cys leu pro gln ile AMB
```

Fig. 8.

```
DNA  sequence   482 b.p.   TGTCTGGCATGG ... CCCCAGATTTAG   linear

9    /   1                                 39   /   11
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu leu pro leu val leu leu glu leu leu val
69   /   21                                99   /   31
GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA
gly ile tyr pro ser gly val ile gly leu val pro his leu gly asp arg glu lys arg
129  /   41                                159  /   51
GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC
glu cys glu ser gly ser phe thr ala ser glu asn his leu arg his cys leu ser cys
189  /   61                                219  /   71
TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC CGG GAC
ser lys cys arg lys glu met gly gln val glu ile ser ser cys thr val asp arg asp
249  /   81                                279  /   91
ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG
thr val cys gly cys arg lys asn gln tyr arg his tyr trp ser glu asn leu phe gln
309  /  101                                339  /  111
TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG
cys phe asn cys ser leu cys leu asn gly thr val his leu ser cys gln glu lys gln
369  /  121                                399  /  131
AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT
asn thr val cys thr cys his ala gly phe phe leu arg glu asn glu cys val ser cys
429  /  141                                459  /  151
AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT TAG
ser asn cys lys lys ser leu glu cys thr lys leu cys leu pro gln ile AMB
```

Fig. 9.

```
DNA  sequence   470 b.p.   TGTCTGGCATGG ... CCCCAGATTTAG   linear

9   /   1                                    39  /  11
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu leu pro leu val leu leu glu leu leu val
69  /  21                                    99  /  31
GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA
gly ile tyr pro ser gly val ile gly leu val pro his leu gly asp arg glu lys arg
129 /  41                                    159 /  51
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
asp ser val cys pro gln gly lys tyr ile his pro gln asn asn ser ile cys cys thr
189 /  61                                    219 /  71
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC
lys cys his lys gly thr tyr leu tyr asn asp cys pro gly pro gly gln asp thr asp
249 /  81                                    279 /  91
TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC
cys arg lys asn gln tyr arg his tyr trp ser glu asn leu phe gln cys phe asn cys
309 / 101                                    339 / 111
AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC
ser leu cys leu asn gly thr val his leu ser cys gln glu lys gln asn thr val cys
369 / 121                                    399 / 131
ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG
thr cys his ala gly phe phe leu arg glu asn glu cys val ser cys ser asn cys lys
429 / 141                                    459 / 151
AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT TAG
lys ser leu glu cys thr lys leu cys leu pro gln ile AMB
```

Fig.10.

```
DNA  sequence   485 b.p.   TGTCTGGCATGG ... CCCCAGATTTAG   linear

9   /   1                                   39  /   11
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu leu pro leu val leu leu glu leu leu val
69  /   21                                  99  /   31
GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA
gly ile tyr pro ser gly val ile gly leu val pro his leu gly asp arg glu lys arg
129 /   41                                  159 /   51
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
asp ser val cys pro gln gly lys tyr ile his pro gln asn asn ser ile cys cys thr
189 /   61                                  219 /   71
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC
lys cys his lys gly thr tyr leu tyr asn asp cys pro gly pro gly gln asp thr asp
249 /   81                                  279 /   91
TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC
cys arg glu cys glu ser gly ser phe thr ala ser glu asn his leu arg his cys leu
309 /   101                                 339 /   111
AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC
ser cys ser lys cys arg lys glu met gly gln val glu ile ser ser cys thr val asp
369 /   121                                 399 /   131
CGG GAC ACC GTG TGT ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC
arg asp thr val cys thr cys his ala gly phe phe leu arg glu asn glu cys val ser
429 /   141                                 459 /   151
TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT TAG
cys ser asn cys lys lys ser leu glu cys thr lys leu cys leu pro gln ile AMB
```

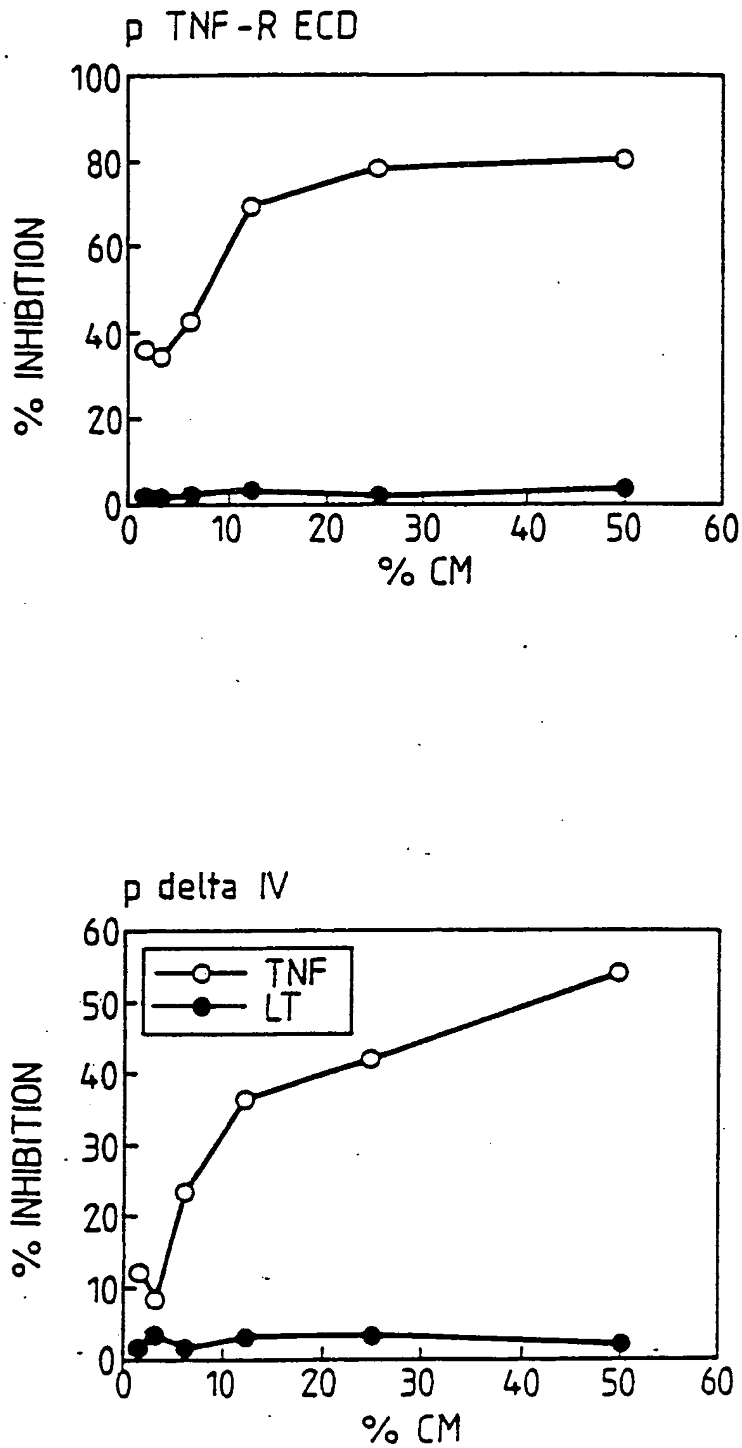
Fig. 11.

```
DNA  sequence    512 b.p.   TGTCTGGCATGG ... GTGTGCACCTGA   linear

9   /   1                                     39  /  11
ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG
met gly leu ser thr val pro asp leu leu leu pro leu val leu leu glu leu leu val
69  /  21                                     99  /  31
GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA
gly ile tyr pro ser gly val ile gly leu val pro his leu gly asp arg glu lys arg
129 /  41                                     159 /  51
GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC
asp ser val cys pro gln gly lys tyr ile his pro gln asn asn ser ile cys cys thr
189 /  61                                     219 /  71
AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC
lys cys his lys gly thr tyr leu tyr asn asp cys pro gly pro gly gln asp thr asp
249 /  81                                     279 /  91
TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC
cys arg glu cys glu ser gly ser phe thr ala ser glu asn his leu arg his cys leu
309 / 101                                     339 / 111
AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC
ser cys ser lys cys arg lys glu met gly gln val glu ile ser ser cys thr val asp
369 / 121                                     399 / 131
CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT
arg asp thr val cys gly cys arg lys asn gln tyr arg his tyr trp ser glu asn leu
429 / 141                                     459 / 151
TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG
phe gln cys phe asn cys ser leu cys leu asn gly thr val his leu ser cys gln glu
489 / 161
AAA CAG AAC ACC GTG TGC ACC TGA
lys gln asn thr val cys thr OPA
```

SUBSTITUTE SHEET

Fig.12.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% CM
p delta IV
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50
40
30
20
10
0
% INHIBITION
TNF
LT
0
10
20
30
40
50
60
% CM